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Composting rapidly reduces levels of extractable oxytetracycline in manure from therapeutically treated beef calves

Osman A. Arikan [a,b,c,*], Lawrence J. Sikora [a], Walter Mulbry [a], Shahamat U. Khan [b], Gregory D. Foster [b]

[a] USDA-ARS, Environmental Management and Byproduct Utilization Laboratory, Building 306, BARC-East, Beltsville, MD 20705, USA
[b] Department of Chemistry and Biochemistry, George Mason University, Fairfax, VA 22030, USA
[c] Department of Environmental Engineering, Istanbul Technical University, Istanbul 34469, Turkey

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Abstract

Oxytetracycline (OTC) is a broad-spectrum antibiotic used in livestock production. The widespread use and relative persistence of OTC may encourage development of antibiotic-resistant bacteria. The objective of this study was to determine whether composting would substantially reduce the concentration of OTC found in manure from medicated animals. The effect of OTC on composting was also investigated. Five beef calves were medicated for 5 days with 22 mg/kg/day of OTC. Approximately 23% of the OTC fed to the calves was recovered in the manure. Manure samples collected from calves prior to and after medication were mixed with straw and woodchips, and aliquots of the subsequent mixtures were treated in laboratory composters for 35 days. In addition, aliquots of the OTC-containing mixture were incubated at 25 °C or sterilized followed by incubation at 25 °C. The presence of OTC did not appear to affect composting processes. Within the first six days of composting, levels of extractable OTC in the compost mixture decreased from 115 ± 8 μg/g dry weight to less than 6 ± 1 μg/g dry weight (a 95% reduction). In contrast, levels of extractable OTC in room temperature incubated and sterilized mixtures decreased only 12–25% after 37 and 35 days, respectively. Levels of total heterotrophic bacteria and OTC-resistant bacteria in the finished compost mixture were roughly 30-fold higher and 10-fold lower, respectively, than levels in the mixture prior to composting. Although the basis of the OTC disappearance during composting is not known, the preponderence of OTC-sensitive bacteria and the decrease of OTC-resistant bacteria in the finished compost suggests that OTC residues have been rendered biologically inactive or unavailable.

Keywords: Oxytetracycline; Composting; Manure; Antibiotic; Calf

1. Introduction

Current livestock production involves the use of large amounts of antibiotics as growth promoters and therapeutic agents. Although both the total and relative amounts of antibiotics used for human and agricultural purposes are not well documented and estimates are controversial (Mellon et al., 2001), one industry group estimates that 9200 tons of antibiotics (including 3000 tons of tetracyclines) were produced in 2003 for both farm and companion animals in the United States (A.H.I., 2005). By comparison, 3900 tons of antibiotics were applied in 1999 in the European Union for veterinary therapy (Thiele-Bruhn, 2003). The manure from the medicated animals is typically managed similarly to that from untreated animals, i.e. it is either stockpiled or immediately applied to farmland as fertilizer. When applied to a field, antibiotic residues in the manure can ultimately reach surface and ground water by run-off or leaching (Halling-Sørensen et al., 1998).

* Corresponding author. Address: USDA-ARS, Environmental Management and Byproduct Utilization Laboratory, Building 306, BARC-East, Beltsville, MD 20705, USA. Tel.: +1 301 504 7327; fax: +1 301 504 8162.
E-mail address: arikano@ba.ars.usda.gov (O.A. Arikan).

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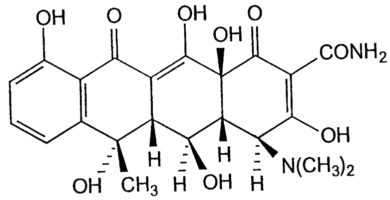

Fig. 1. Chemical structure of oxytetracycline (OTC) (Molecular weight: 460.44, pK_a: 3.3/7.3/9.1).

Oxytetracycline (OTC) (Fig. 1) is administered to animals to control intestinal and respiratory infections. Considerable research has been carried out on the detection of OTC in edible tissues (Fedeniuk et al., 1996; Capolongo et al., 2002), on the degradation of OTC in sediments (Samuelsen, 1989; Björklund et al., 1990; Hektoen et al., 1995; Capone et al., 1996; Coyne et al., 2001), in simulated surface water systems (Ingerslev et al., 2001), and in soil interstitial water (Halling-Sørensen et al., 2003). OTC persists in the sediment for several months (Björklund et al., 1990; Hektoen et al., 1995) and has been detected (at levels averaging 9 µg/kg) in the upper 10 cm of soil from fields that had been sprayed with manure slurry two days prior to sampling (Hamscher et al., 2000). Of primary concern is the detection of OTC in surface water samples, albeit at very low concentrations (ranging from 0.07 to 1.34 µg/l) (Lindsey et al., 2001). In addition, the persistence of OTC residues in the environment may contribute to bacterial resistance to tetracyclines (Witte, 2000).

Treatment of manure containing OTC is one possible means of reducing the amount of this compound that is ultimately released into the environment. However, there is very limited scientific information on the fate and effect of OTC during manure treatment processes. Composting is a treatment process that is increasingly integrated into manure management operations. Although there is no information on the effect of composting on tetracyclines and their residues, a variety of studies have shown significant reductions of other relatively persistent organic compounds such as 2, 4, 6-trinitrotoluene (Griest et al., 1993), diazinon (Michel et al., 1997), pyrene and simazine (Hartlieb et al., 2003), 17β-estradiol and testosterone (Hakk et al., 2005). The objectives of this study were two-fold: to determine whether OTC residues in manure from treated animals inhibit composting microbial processes and to determine the effect of composting on levels of extractable OTC in manure.

2. Methods

2.1. Animal medication and sample collection

Five male beef calves, 4–6 months old and ranging from 170 to 240 kg in body mass, were kept in individual pens at the Beltsville Agricultural Research Center Beef Barn. Pens were scraped clean daily, after which approximately 2 kg of sawdust was scattered on the floor of each pen as bedding material. After a two-week acclimatization period for the animals, the manure-sawdust mixture from each pen was collected (averaging 15 kg/animal-day), pooled, mixed, and 75 kg of this mixture was stored at 4 °C until later use as the "unmedicated" manure. The calves were then medicated for 5 days at 22 mg/kg body mass per day of OTC (a standard dosage in agricultural practice) by ingestion of the daily ration containing OTC as a feed additive. This corresponded to daily doses of 3.7–5.3 g of OTC per animal with an average value of 23.1 g for five calves per day. Feed consisted of a mixture of unmedicated Ncf2 beef creep pellet (9%), sudan silage (17%), corn silage (24%), and medicated Ncf2 beef creep pellet (50%). Medicated grain was given to the animals prior to other constituents in order to insure complete consumption of the OTC dose. In order to determine excretion profiles of OTC from treated calves, pooled medicated manure-bedding mixtures from all calves were collected during the 5 days of medication followed by two additional collections on days 7 and 9. Medicated manure-bedding mixtures collected on the fifth day of medication (when OTC levels were expected to be highest) were combined and used in laboratory composting experiments. Table 1 shows characteristics of the unmedicated and medicated manure-bedding mixtures.

Table 1
Characteristics of medicated and unmedicated manure-bedding mixtures from animals prior to and after OTC medication

Constituent	Medicated manure-bedding mixture	Unmedicated manure-bedding mixture
Moisture content (%)	72.4 ± 0.2	74.3 ± 0.3
C (%)	12.9 ± 0.2	11.8 ± 0.1
N (%)	0.4 ± 0.1	0.5 ± 0.1
C/N	29	26
Total phosphorus (mg/g) DW	6.8 ± 0.4	9.3 ± 2.1
Ammonia (mg/g) DW	1.9 ± 0.1	4.1 ± 0.1

Mean ± std error.

2.2. Composting experiments

Composting studies were conducted in self-heating laboratory composters (Sikora et al., 1983). Each composter is comprised of a covered, double walled, insulated tank; an air-tight cylinder; an inner screen mesh cylinder (8400 cm^3 capacity) that contains the material to be composted; a heater, and a differential temperature control system (Fig. 2). Aeration was controlled by a rotameter and the temperature differential between the composted material and the insulated tank was set at 2–3 °C. A portion of the pooled manure-bedding mixture collected from calves on the fifth day of medication was mixed with straw and hardwood woodchips in a ratio of 3:1:1 (v/v) and 3.3 kg aliquots of the resulting mixture were loaded into each of four cylinders and placed into composters (composters 1–4). A comparable mixture of unmedicated manure-bedding (collected

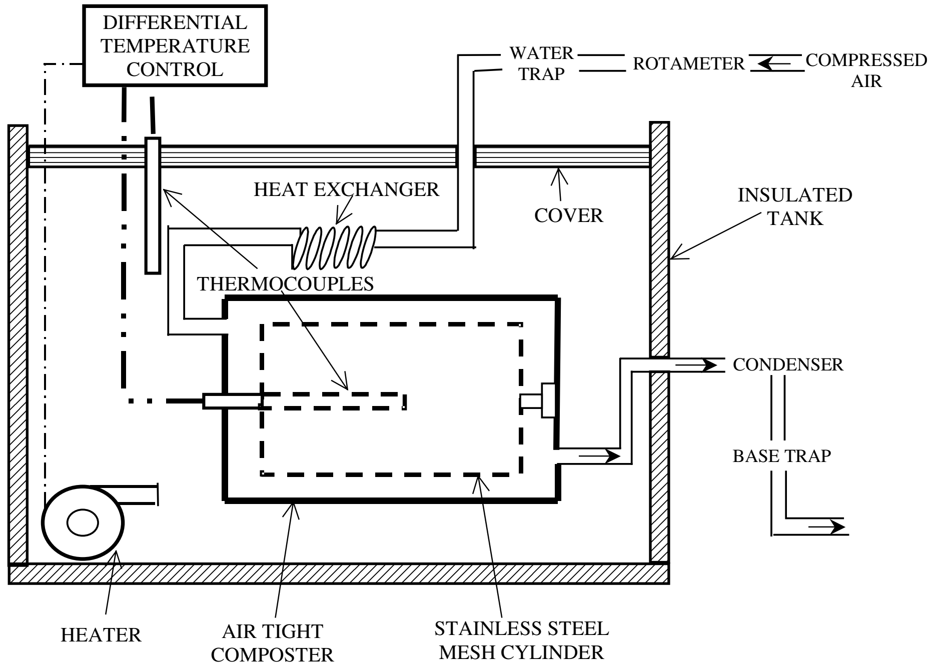

Fig. 2. Schematic illustration of the self-heating laboratory composting system.

from animals prior to OTC medication), straw, and woodchips were loaded into a fifth cylinder and placed into a separate composter (composter 5).

Prior experiments with these self-heating composters have shown that multiple samplings during incubation can lead to significant disruptions in CO_2 and temperature profiles (Sikora, unpublished results). In order to avoid such potential disruptions, individual composters were sampled only once during the 35 day composting period. The sampling dates for each composter were: day 0 (composters 1–5), day 6 (composter 1), day 16 (composter 4), day 19 (composter 2), day 28 (composter 3), and, day 35 (composters 1–5). At each time point, the composting mixture to be sampled was unloaded from the composter cylinder into a bucket, mixed prior to removal of 100 g samples, and the remaining material was reloaded into the cylinder and respective composter.

Composter temperatures were recorded every 15 min using a datalogger. Carbon dioxide evolved from each composter was trapped using 6 N NaOH trap solutions that were changed daily. Carbon dioxide levels were determined by titration using 0.2 M H_2SO_4 and phenolphthalein as an indicator after precipitation of carbonate with $BaCl_2$ (Zibilske, 1994). Composter 4 was aerated, but temperature and CO_2 parameters were not monitored. For moisture determination, manure and compost samples were dried for 24 h at 105 °C. The organic C and N contents of samples were determined using the catalytic tube combustion method and a Vario Max CNS Macro Elemental Analyzer with a TCD detector (Elementar Americas, Inc., Mt. Laurel, NJ). Total phosphorus content was determined by block digestion and flow injection analysis (Lachat Instruments, Milwaukee, WI). For ammonium–N analysis, 5 g samples were extracted with 50 ml 2 M KCl on a rotary shaker for 60 min. The resulting extracts were filtered, adjusted to pH 5 with H_2SO_4, and stored at 4 °C until analysis. Ammonium–N was determined colorimetrically by flow injection analysis (Lachat Instruments, Milwaukee, WI).

The significance of OTC's effect on cumulative CO_2 evolution during composting was determined with the repeated measures ANOVA procedure using SAS Proc Mixed (SAS Institute Inc., 2004). Significant differences were determined at the $p < 0.05$ level of significance.

2.3. Room temperature incubation and sterilization of OTC-containing compost mixture

Duplicate 50 g aliquots of the OTC-containing compost mixture (described above) were placed in separate 250 ml flasks, and incubated at room temperature (23–27 °C) for 37 days (referred to hereafter as the room temperature incubated mixture). In order to determine the effect of abiotic processes on OTC levels in manure, a 280 g aliquot of OTC-containing compost mixture was placed in a 500 ml flask, sterilized by irradiation (3 Mrads for 395 min from a Co^{60} source at the University of Maryland Radiation Facilities, College Park, MD), sealed with a rubber stopper, and incubated at room temperature (referred to hereafter as the sterilized mixture). Samples were taken from the room temperature incubated and sterilized mixtures at the beginning (day 0) and at the end of the incubations (day 35 for the sterilized mixture and day 37 for the room temperature incubated mixture).

2.4. Extraction and analysis of OTC

Manure and compost subsamples were extracted in duplicate for OTC analysis using the method described by Capone et al. (1996). Briefly, 1 g subsamples were extracted three times with 3 ml of 0.1 M Na_2EDTA-McIlvaine buffer by vortexing for 30 s followed by sonication for 3 min in a 100 W sonication bath (Bronson Ultrasonics, Danbury, CT). After each extraction, the extracts were subjected to centrifugation (500*g*, 5 min, 5 °C), the supernatants pooled, again subjected to centrifugation (1650*g*, 20 min, 5 °C), filtered through Whatman 5 filter paper, and passed through Waters Sep-Pak C-18 cartridges after the cartridges had been previously flushed with 5 ml methanol and 10 ml 0.1 M Na_2EDTA-McIlvaine buffer. After extracts were loaded, cartridges were flushed with 20 ml distilled water, followed by sample elution using 8 ml of 0.01 M methanolic oxalic acid. Eluents were concentrated to approximately 1 ml by evaporation prior to OTC analysis by isocratic HPLC using an Agilent Technologies HP 1100 system (Sunnyvale, CA), a 25 cm Partisil 10 ODS column, a mobile phase consisting of acetonitrile–0.01 M oxalic acid (25:75, v/v) at a flow rate of 1 ml min^{-1}, 20 µl sample injections, and detection at 365 nm. Calibration curves were typically generated using results from 20 µl injections of OTC standards ranging from 0.1 µg/ml to 10 µg/ml.

To determine OTC extraction efficiencies, triplicate samples of three matrices (water, manure-bedding and manure-bedding–straw–woodchips) were spiked to contain 1 and 25 µg/ml or µg/g wet weight of OTC, and extracted as above prior to OTC analysis.

2.5. Estimation of total heterotrophic and OTC-resistant bacteria in the composted mixture

Levels of total heterotrophic and OTC-resistant bacteria in the OTC-containing manure-bedding–straw–woodchip mixture before and after 35 days of composting were determined by standard dilution plating using nonselective Luria Bertani (LB) media and LB media containing 30 µg ml^{-1} OTC, respectively. Bacterial counts were determined after incubation at 30 °C for 48 h. All analyses were conducted using duplicate samples.

3. Results

3.1. Analysis and extraction recovery of OTC

Using the extraction and HPLC conditions described above, the retention time for OTC was 5.6 ± 0.1 min, the calibration curves were linear with correlation coefficients >0.99, and the detection limit of OTC in samples was 0.24 µg/g DW (dry weight). Recovery results for OTC shown in Table 2 were calculated as means of triplicate samples at two concentrations in water, manure-bedding and manure-bedding–straw–woodchips mixtures. Although recoveries of OTC from the manure-bedding and manure-bedding–straw–woodchips mixtures were significantly lower than that from distilled water, recovery of OTC was greater than 70% in each of these matrices and appeared to be independent of initial concentration.

Table 2
Extraction efficiencies for OTC in different matrices

Matrix	Extraction efficiency (% mean ± std error)	
	Spike level	
	1 µg/ml or µg/g wet weight	25 µg/ml or µg/g wet weight
Water (*n* = 3)	95 ± 7	93 ± 4
Manure-bedding (*n* = 3)	71 ± 9	72 ± 9
Manure-bedding–straw–woodchips (*n* = 3)	72 ± 8	76 ± 6

3.2. Excretion profiles of OTC from treated calves

The relationship between the quantity of OTC administered to the calves and extractable OTC measured in the collected manure is shown in Fig. 3. The daily amount of extractable OTC in the collected manure peaked on the second day, decreased gradually during the remaining medication period, and then decreased rapidly after medication ceased. Roughly 23% of the administered OTC was recovered within the first nine days after treatment began. Although the sample size is quite small, there was little variation between the calves with respect to their OTC excretion rates (data not shown).

3.3. Effect of OTC on composting processes

All composters reached temperatures exceeding 65 °C within 2–3 days following start-up (Fig. 4a). Temperatures gradually declined to 40–50 °C during the following two weeks, then remained relatively constant until the fourth

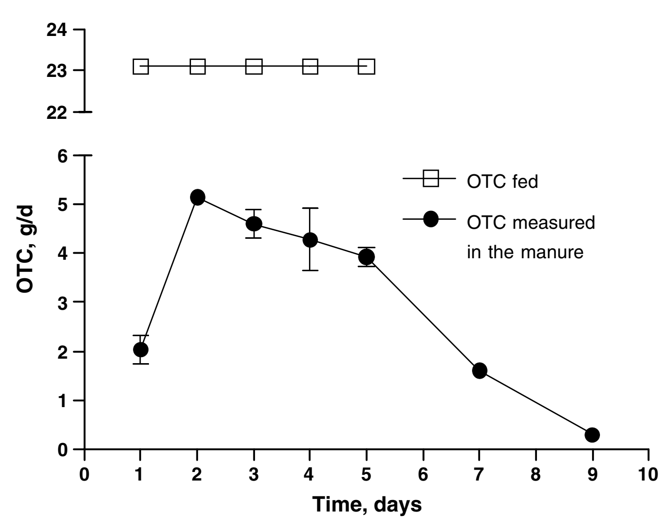

Fig. 3. Calculated total quantities of OTC fed to five calves and total extractable OTC measured in the collected manure (mean ± std error) over time.

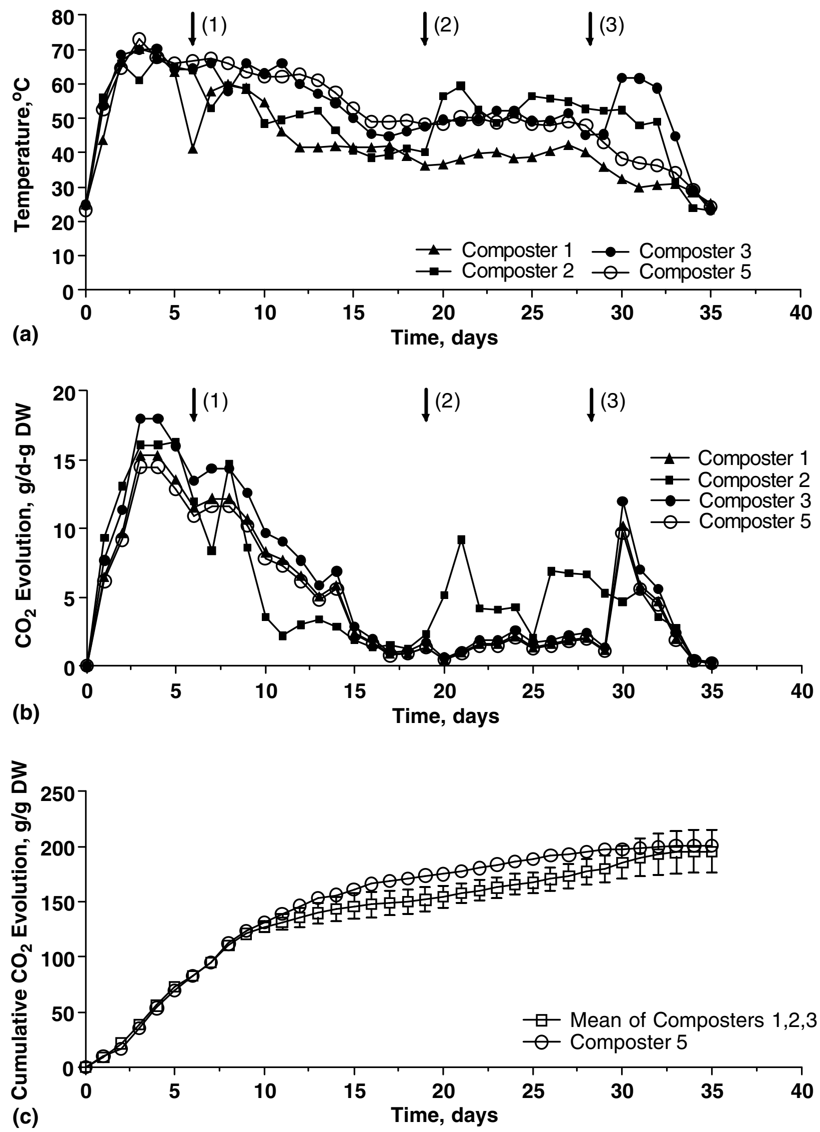

Fig. 4. Comparison of temperature, daily and cumulative CO_2 evolution from composters containing the medicated and the unmedicated manure mixtures during composting: (Panel a) daily temperature; (Panel b) daily carbon dioxide evolution; (Panel c) cumulative carbon dioxide evolution. Composters 1–3 contain the medicated manure mixture. Composter 5 contains the unmedicated manure mixture. Arrows indicate the sampling date and numbers in brackets show the composter sampled.

week of incubation whereupon temperatures decreased to 25–30 °C by day 35. The initial rapid increase in temperature and high temperatures observed during composting are due to high levels of microbial activity. Carbon dioxide evolution from composters followed temperatures pattern closely. Maximum carbon dioxide evolution coincided with peak temperatures, and gas evolution declined as temperatures decreased (Fig. 4b). Peaks in both temperature and carbon dioxide evolution were observed immediately after sampling and were attributable to mixing. The mean cumulative carbon dioxide evolution (Fig. 4c) for composters 1–3 (containing the medicated-manure mixture) and composter 5 (containing the unmedicated manure mixture) were not significantly different ($p > 0.05$). In addition, there were no significant differences between the medicated and unmedicated mixture after composting with respect to changes in pH, moisture content and C:N ratio (not shown). These results suggest that levels of OTC in the compost mixtures did not affect the composting process.

3.4. Extractable OTC levels in composted, room temperature incubated and sterilized mixtures

The extractable OTC concentration in the manure-bedding mixture (collected on the fifth day of medication) was 225 ± 15 μg/g DW. The initial concentration of extractable OTC in the mixture to be composted was approximately 50% lower (115 ± 8 μg/g DW) due to the addition of straw and woodchips. The extractable OTC level in the composted mixture decreased to 6 ± 1 μg/g DW within six days and subsequently decreased to levels below the detection limit (0.24 μg/g DW) by the end of the 35-day composting

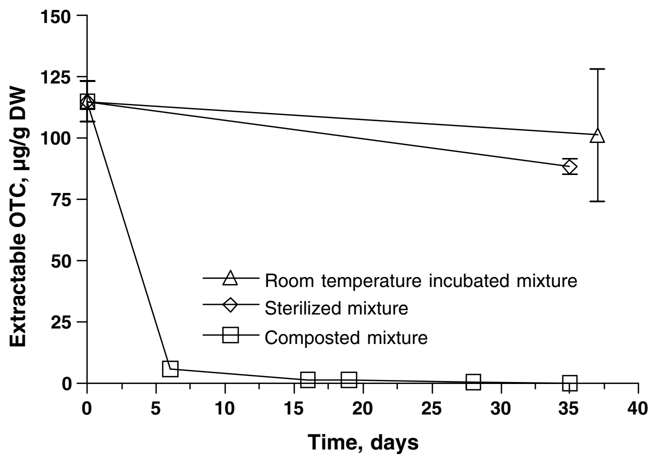

Fig. 5. Extractable OTC levels as a function of incubation period for the composted mixture, room temperature incubated mixture, and sterilized mixture (mean + std error). The initial extractable OTC concentration within these mixtures was 115 ± 8 µg/g DW.

experiment (Fig. 5). Overall, more than 99.8% removal of extractable OTC was achieved during composting. In contrast, levels of extractable OTC in room temperature incubated and sterilized aliquots of the manure-bedding–straw–woodchip mixture decreased only 12% (to 101 ± 27 µg/g DW) and 25% (to 88 ± 3 µg/g DW), after 37 days and 35 days of incubation at room temperature, respectively (Fig. 5). Although the mean value for OTC removal in the room temperature incubated mixture was lower than that in the sterilized mixture, this is likely due to the high variability observed in samples of the room temperature incubated mixture.

3.5. Effect of composting on levels of total heterotrophic and OTC-resistant bacteria

Total heterotrophic and OTC-resistant bacteria in the manure–straw–woodchips mixture before and after 35 days of composting were estimated as a biological measure of the concentration and bioavailability of OTC residues. Total heterotrophic bacterial levels (CFU/g DW) increased 30-fold from 2.3×10^8 ($\pm 1.2 \times 10^8$) prior to composting to 7.6×10^9 ($\pm 3.5 \times 10^8$) at day 35. In contrast, levels of OTC-resistant bacteria decreased nearly 10-fold from 2.1×10^7 ($\pm 2.1 \times 10^6$) prior to composting to 2.8×10^6 ($\pm 9.5 \times 10^5$) at day 35.

4. Discussion

Our OTC recovery values of roughly 70% from manure-bedding and manure-bedding–straw–woodchips mixtures are comparable to values reported by others. De Liguoro et al. (2003) reported OTC recoveries of roughly 80% using calf manure and manure-bedding mixtures. Blackwell et al. (2004) developed a ultrasonic extraction method that resulted in OTC recoveries of 77–102% from pig slurry and 27–75% recoveries from slurry amended soils. Capone et al. (1996) reported OTC recoveries of 50–70% from sediments and 65–80% from crab and oyster tissue. Comparison of the recoveries from distilled water and antibiotics added to the manure matrices indicates that the low recovery from the manure may be due to the sorption or complexation binding of OTC to particles in the manure (Table 2).

Approximately 23% of the OTC fed to the calves was recovered in the manure. Previous studies have reported that wethers (castrated sheep) excreted at least 21% of the oral dose of OTC and that young bulls excreted 17–75% of an oral dose of chlorotetracycline (cited in Montfords et al., 1999). Winckler and Grafe (2001) reported tetracycline excretion rates for swine ranging from 42% to 72% depending on dose.

In our study, the calculated half-life of OTC in composted manure-bedding–straw–woodchips mixture was approximately 3.2 days. The fate of tetracyclines in various matrices has been the subject of numerous studies. De Liguoro et al. (2003) observed the half-life of OTC in stockpiled manure-bedding mixture was 30 days and the compound was still detectable in this matrix (820 µg/kg) after 5 months. Winckler and Grafe (2001) reported a half-life of tetracycline in spiked pig slurry ranged between 55 and 105 days. In contrast, Kühne et al. (2000) reported half-life values for tetracycline in aerated and nonaerated pig manure of 4.5 and 9 days, respectively. Field studies have reported OTC half lives in sediments of 12 and 15 days (Coyne et al., 2001), 32 days (Samuelsen, 1989), 151 days (Hektoen et al., 1995), 9–419 days (Björklund et al., 1990) and 36 days in laboratory microcosms (Capone et al., 1996).

There was a rapid reduction of extractable OTC concentrations within the first six days of composting. However, our experiments do not resolve whether the reduction of extractable OTC is caused by degradation, mineralization or binding of OTC to the compost matrix. In addition (and in common with numerous other studies focused on the degradation of xenobiotics during composting) these experiments do not separate the relative contributions of individual factors that occur during composting (elevated temperatures, high biological activity, biologically transformed organic material) that affect levels of extractable OTC nor the interactions between these factors. The purpose of using a sterilized mixture in our study was to determine the extent of OTC removal due only to sorption to the manure-bedding–straw–woodchips mixture. Based on the reduction of extractable OTC levels in the sterilized mixture over 35 days at 25 °C, OTC removal due only to sorption was probably not significant relative to other removal processes during the first six days of composting. Thiele-Bruhn and Beck (2005) showed 90%, 70% and 60% decline of the extractable OTC in a 14 day soil incubation study at 25 °C using 10 µg/g, 100 µg/g and 1000 µg/g spiking levels, respectively. They suggested that the decline of extractable OTC levels was likely due to non-linear adsorption of OTC. Strong sorption of OTC to solid matrices was found by Rabølle and Spliid (2000), Loke et al. (2002), Pouliquen and Le Bris (1996). The temperature dependency of the rate of tetracycline disappearance has been previously shown in a soil-chicken faeces mixture by Gavalchin and Katz (1994). They found that 44%, 88%, and 100% of chlortetracycline remained in soil-chicken faeces mixture after 30 days of incubation at 30 °C, 20 °C and 4 °C, respectively. Hartlieb et al. (2003) suggested that adsorption sites are generated during composting. They found that 30% of simazine residues became bound within a non-extractable fraction after the thermophilic stage of composting (29 days after the start of composting in their study). In the same study, 23.8% of pyrene became non-extractable and 60% was mineralized after 370 days of composting.

In the absence of definitive knowledge on the fate of OTC and its metabolites within the composted matrix, we measured the populations of total heterotrophic and OTC-resistant bacteria before and after composting. Microbial population counts show greatly increased numbers of total heterotrophic organisms and decreased numbers of OTC-resistant organisms after composting of the OTC-containing mixture. Although this result suggests that non-extractable OTC and OTC metabolite residues are not bioactive, we do not yet have any genetic information about the OTC resistant organisms. Since a primary concern is focused on the transfer of antibiotic resistance within the soil microbial flora, it would be beneficial to characterize the OTC-resistant populations and the responsible genetic elements prior to and after composting. Such characterization would increase our understanding of whether resistant microbial populations in manure from treated animals survive manure treatment and/or transfer genetic elements to other organisms.

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